

## Detection of Simian Immunodeficiency Virus (SIV)-Specific CD8<sup>+</sup> T Cells in Macaques Protected from SIV Challenge by Prior SIV Subunit Vaccination

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Received 27 December 1995/Accepted 30 April 1996

Vaccines for lentiviruses would ideally induce in the host complete resistance to infection of host cells. However, such sterilizing immunity may be neither readily achievable nor absolutely necessary to provide protection from exposure to the immunodeficiency viruses. To examine the nature of protective immunity to simian immunodeficiency virus (SIV), we studied three macaques that had been immunized with a recombinant vaccinia virus-based SIV subunit vaccine regimen and exhibited protection from a challenge with cell-free SIV<sub>MNE</sub> as determined by viral cultures, serology, and PCR for viral genomes. Peripheral blood mononuclear cells were obtained from the protected macaques and analyzed for CD8<sup>+</sup> cytotoxic T-lymphocyte (CTL) responses to SIV proteins. CTL reactive to SIV proteins not included in the subunit vaccine, and thus to which these animals had not been exposed prior to challenge, were detected postchallenge in the vaccine-protected animals and persisted for up to 1 year. These CTL, as reflected by studies of cytolytic lines and derived T-cell clones, were CD8<sup>+</sup>, did not recognize allogeneic targets, and recognized the SIV proteins in the context of class I major histocompatibility complex molecules. The frequency of precursor CD8<sup>+</sup> CTL reactive to SIV proteins was determined by limiting-dilution analysis and demonstrated that the responses elicited following challenge of protected animals to SIV proteins not present in the vaccine were quantitatively similar to those of animals persistently infected with SIV. The presence of these CD8<sup>+</sup> CTL responses to SIV proteins present only in the challenge virus suggests that infection of some host cells occurred postchallenge. These results suggest that the development of a low level of SIV infection following exposure of vaccinated hosts to SIV does not preclude protection from lethal SIV disease by vaccine-induced immunity.

Recombinant vaccines containing viral subunits have been demonstrated to be both safe and effective for several viral diseases (3, 32). Optimally, a vaccine should induce sterilizing immunity that completely prevents infection of host cells following exposure to the virus. Although prevention of infection has been observed in experimental settings in some animal models (33), human viral vaccines have generally been found to be effective not by inducing sterilizing immunity but rather by limiting infection and preventing disease (8, 19). For viruses like human immunodeficiency virus type 1 (HIV-1), the potential adequacy of vaccines that induce such nonsterilizing responses for containing infection has been questioned, since the burst of acute viral replication that would follow even limited infection could lead to integration of proviral DNA into the host genome, resulting in the establishment of latency in some infected cells, the development of intermittent viral activation and associated persistent infection, and ultimately progressive immunodeficiency (6, 9, 31, 36). However, recent studies demonstrating the presence of CD8<sup>+</sup> cytotoxic T lymphocytes (CTL) reactive with peptides derived from HIV-1 proteins expressed in infected cells in the peripheral blood of seroneg-

ative humans following sexual or perinatal HIV-1 exposure have suggested that transient and clinically silent HIV-1 infections may occur (4, 24, 34, 35). While these observations provide optimism that lentivirus vaccines, similar to other human viral vaccines, might be capable of inducing protective responses without the requirement for generating sterilizing immunity, formal proof supporting this hypothesis has not been evident.

The simian immunodeficiency virus (SIV)-macaque model has proven useful for evaluating candidate lentivirus vaccines (26, 38). Although the precise mechanism(s) by which SIV is eliminated from SIV-challenged macaques is unknown, immunization to SIV subunits, including priming with a live recombinant vaccinia virus (rVV) and boosting with a recombinant protein, has been shown to protect macaques from subsequent challenge with a pathogenic clone of SIV<sub>MNE</sub> (14, 15, 17). In this report, we examine, by evaluating the nature of the immune responses present following exposure to infectious virus, if this protection necessarily reflects prevention of infection of host cells. All macaques evaluated had been vaccinated and monitored postchallenge for evidence of infection. None of the three study animals had virus isolatable from peripheral blood mononuclear cells (PBMC) at any time following SIV challenge or evidence of seroconversion to SIV proteins, consistent with the perception that infection had been prevented. However, an analysis of CD8<sup>+</sup> CTL responses postchallenge revealed the induction of reactivity to SIV proteins to which the animals had not been previously exposed but which are known to be expressed in an immunogenic fashion in infected cells

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TABLE 1. Vaccination regimen and experimental challenge of three vaccine-protected macaques<sup>a</sup>

Animal no.	SIV <sub>MNE</sub> vaccine regimen	Times (wk) vaccine given	Route and time (wk) of SIV challenge <sup>b</sup>	Challenge virus (animal infectious doses)	SIV isolation postchallenge	SIV seroconversion postchallenge	SIV DNA in PBMC postchallenge	SIV DNA in lymph nodes postchallenge
87217	Vac-env	0, 12	i.v. (142) <sup>c</sup>	SIV <sub>MNE</sub> uncloned (10-99)	Negative	Negative	Negative	Negative
90071	gp160 in IFA <sup>d</sup>	62, 70, 139	i.r. (93)	SIV <sub>MNE</sub> clone E11S (2-18)	Negative	Negative	Negative	Not tested
91278	Vac-gag/pol	0	i.v. (111)	SIV <sub>MNE</sub> clone E11S (10-99)	Negative	Negative	Week 2 only <sup>e</sup>	Negative
	gag/pol in IFA	10, 84, 89						
		0						
		12, 74, 104						

<sup>a</sup> The challenge virus was grown in Hut-78 cells for animals 87217 and 90071 and in macaque PBMC for animal 91278.

<sup>b</sup> Animals were challenged with infectious SIV<sub>MNE</sub> by either intravenous (i.v.) injection or intrarectal (i.r.) inoculation following mucosal abrasion.

<sup>c</sup> Animal 87217 had previously resisted to an intravenous challenge at week 74 with 1 to 9 animal infectious doses of cloned SIV<sub>MNE</sub> (17) prior to the challenge at week 142 with uncloned SIV<sub>MNE</sub> evaluated in this report.

<sup>d</sup> IFA, incomplete Freund adjuvant.

<sup>e</sup> PBMC from animal 91278 were positive in three samples obtained 2 weeks postchallenge for 40 copies of SIV DNA per µg of input DNA measured by semiquantitative PCR (13). By comparison, three unvaccinated animals similarly challenged with SIV had a mean of 2,000 copies of SIV DNA per µg of DNA at 2 weeks postinfection.

and thus has provided evidence supporting the presence of a transient infection postchallenge.

#### MATERIALS AND METHODS

**Animals.** All macaques (*Macaca fascicularis*) were cared for at the University of Washington Regional Primate Research Center (accredited by the American Association for Accreditation of Laboratory Animal Care) and anesthetized with ketamine (10 mg/kg intramuscularly) prior to procedures. Three vaccinated animals were selected for study following the demonstration, as described below, over a prolonged time period of protection from a virulent SIV challenge (Table 1). Two SIV subunit vaccine regimens with rVV priming and recombinant protein boosting, based on immunization either to SIV envelope (gp160) protein from strain SIV<sub>MNE</sub> (15) or to SIV gag/pol-encoded protein from strain SIV<sub>MNE</sub>, were used in these studies. The recombinant vaccine proteins used for immunization were purified following production in either baculovirus-infected insect cells (animal 87217 only) (14) or rVV-infected mammalian cells. Twelve animals enrolled in three separate experiments were vaccinated and challenged; 5 of these 12 animals were subsequently shown to be protected from SIV challenge and thus potentially evaluable, but only 3 of these 5 protected animals were available for analysis of CTL responses. The first animal (animal 87217) was selected from an experiment examining the breadth of viral isolates encompassed by protective immunity, in which 4 animals previously vaccinated to SIV<sub>env</sub>, which demonstrated resistance to an initial intravenous challenge with a cloned SIV<sub>MNE</sub> isolate (14), were subsequently rechallenged with uncloned SIV<sub>MNE</sub>. Only the one animal evaluated, animal 87217, resisted challenge with uncloned SIV<sub>MNE</sub>. The second animal (animal 90071) was selected from an experiment assessing the impact of route of exposure on vaccine-induced protection, in which three of four macaques previously immunized to SIV<sub>env</sub> resisted intrarectal challenge with cloned homologous SIV<sub>MNE</sub>; only one of the three animals, animal 90071, was available for this study (27, 39). The third animal was selected from an experiment assessing if immunization to SIV gag/pol could induce protective immunity, in which four animals previously immunized to SIV gag/pol were challenged with cloned homologous SIV<sub>MNE</sub>; the one animal demonstrating protection, animal 91278, was studied (16).

The challenge virus inoculated by either the intravenous or intrarectal route was obtained from cell-free SIV<sub>MNE</sub> stocks titrated in macaques (clone E11S, homologous to the vaccine strain, or uncloned SIV<sub>MNE</sub>) that had been grown in human Hut-78 cells or macaque PBMC (13, 14). Animals were monitored for SIV infection at 2, 4, 8, 12, 16, and 20 weeks postchallenge and at four to eight weekly intervals thereafter for 60 to 160 weeks by three separate assays for reverse transcriptase activity following coculture of PBMC with phytohemagglutinin-stimulated cells, the presence of SIV proviral DNA in PBMC by PCR using nested primers, and seroconversion to nonvaccine SIV proteins in serum by immunoblotting as previously described (14). Additionally, mononuclear cells obtained from inguinal lymph node biopsies at week 20 (animal 91278) and week 43 (animal 87217) post-SIV challenge were assayed for proviral DNA by PCR.

The sensitivity and specificity of the assays used in these studies to detect SIV infection have been previously defined at our center (14, 15, 17, 18, 23). Briefly, for SIV coculture, in samples from >16 unvaccinated control SIV<sub>MNE</sub>-infected animals, >90% of PBMC samples were coculture positive for SIV up to 8 weeks postinfection, with all animals positive for virus isolation on multiple occasions. After 8 weeks, approximately 50% of coculture samples from SIV<sub>MNE</sub>-infected animals were positive for virus isolation. For the SIV DNA PCR detection system used, the assay was sensitive for >1 to 3 copies of SIV per µg of input DNA. In all unvaccinated control SIV<sub>MNE</sub>-infected animals studied at the Uni-

versity of Washington (>100 animals), SIV DNA is detected by PCR in every sample taken up to the time of death, with the peak level of SIV DNA occurring at 2 weeks postinfection (the earliest time point sampled). Uninfected control animals are uniformly negative for SIV DNA by PCR. The enzyme immunoassay and Western blot (immunoblot) methods that were used to detect a seroconversion with titers of 1:10,000 to 1:100,000 in unvaccinated SIV<sub>MNE</sub>-infected control animals and reveal titers of <1:25 or <1:100 (the lowest dilutions used) in uninfected control animals. For lymph node analysis of SIV DNA by PCR, lymph node mononuclear cells from control SIV<sub>MNE</sub>-infected macaques (>10 animals) were uniformly positive by PCR for SIV, and lymph nodes sampled from uninfected control macaques (>4 animals) were uniformly negative by PCR for SIV.

**Generation of CTL.** Effector cells were generated and tested as previously described (22). Briefly, fresh PBMC (10<sup>6</sup> cells per ml in RPMI with 10% human AB<sup>+</sup> serum) were cultured at a responder/stimulator (R/S) ratio of 10:1 for 1 week with autologous PBMC that had been infected for 16 h with the recombinant fowlpox viruses rFPV<sub>SIV/MAC251env</sub> and rFPV<sub>SIV/MAC251gag/pol</sub> at a multiplicity of infection (MOI) of 25:1 and for a second week with both autologous γ-irradiated (8,000 rad) herpesvirus papio-transformed B-lymphoblastoid cell lines (BLCL) infected with the same rFPV constructs (R/S ratio = 10:1) and fresh autologous γ-irradiated (3,000 rad) PBMC (R/S ratio = 1:1) as filler cells. Two days after the second stimulation, 2 to 4 U of recombinant interleukin-2 (rIL-2; Hoffman-LaRoche, Nutley, N.J.) per ml was added. At the end of the second week of culture, responding cells were harvested and tested against autologous BLCL target cells infected with rVV containing either no inserted genes (Vac) or SIV<sub>MNE</sub> env (Vac-env) or SIV<sub>MNE</sub> gag/pol (Vac-gag/pol) genes for 16 h (MOI = 10:1). Targets were labeled with 0.1 µCi of Na<sup>251</sup>CrO<sub>4</sub> (NEN Products) and plated at 5 × 10<sup>3</sup> cells per well in 96-well U-bottom plates at various effector/target (E/T) ratios. After a 4-h incubation of effectors and targets, supernatant was sampled and counted. Percent specific lysis was calculated by the standard formula, with spontaneous release determined from targets cultured in media alone and maximum release determined from targets lysed with 1% Nonidet P-40 detergent. The standard deviation of triplicate wells was <6%, and spontaneous release was <28% of the maximal release.

**pCTL determinations.** CTL precursor (pCTL) analyses were performed as a modification of previously described methods (21). Briefly, fresh PBMC, or subpopulations of fresh PBMC positively and negatively selected on an anti-CD8 (αCD8) monoclonal antibody (MAb)-coated flask, were stimulated at various dilutions (3 × 10<sup>4</sup> to 5 × 10<sup>5</sup> cells per well in 24 replicates) with autologous γ-irradiated (8,000 rad) BLCL (10<sup>4</sup> cells per well) infected with rFPV expressing SIV<sub>env</sub> and gag/pol (MOI = 25:1) and rIL-2 (25 U/ml added every 3 days) for 10 days. Cells were harvested and tested for lytic activity against a panel of autologous rVV-infected BLCL targets. Wells demonstrating target lysis exceeding background lysis with medium alone by >3 standard deviations were scored positive.

**Separation of CD4<sup>+</sup> and CD8<sup>+</sup> T-cell subsets.** Positive and negative selection to acquire CD8<sup>+</sup> and CD4<sup>+</sup>-enriched cell fractions was performed as previously described (21, 22) by panning on an αCD8 MAb-coated flask (Applied Immune Sciences, Menlo Park, Calif.). Fluorescence-activated cell sorting (FACS) analysis of cell populations stained with αCD4 and αCD8 MAbs (Leu3a and Leu2a; Becton Dickinson) demonstrated that the unseparated population of in vitro-cultured PBMC contained 71% CD8<sup>+</sup> cells and 22% CD4<sup>+</sup> cells, the CD4-enriched effector cell population contained 87% CD4<sup>+</sup> cells and 2% CD8<sup>+</sup> cells, and the CD8-enriched population contained 97% CD8<sup>+</sup> cells and <1% CD4 cells.

**Cloning of CTL.** Cells from cultures of PBMC demonstrating SIV gag/pol-specific lytic activity were plated at 0.3 cells per well, and each well was stimu-

lated with autologous  $\gamma$ -irradiated PBMC ( $10^5$  cells per well), autologous UV- and  $\gamma$ -irradiated BLCL ( $10^4$  cells per well) previously infected for 16 h with an rVV expressing SIV<sub>MNE</sub> *gag/pol* (MOI = 10:1), and 50 U of rIL-2 per ml as previously described (21). Wells demonstrating cell growth were expanded, phenotyped with fluoresceinated antibodies by flow cytometry, and assessed for lytic activity against autologous and allogeneic targets.

**Analysis of class I restriction of CTL responses.** Since the polyclonal CTL lines generated in vitro should contain responses to the multiple major histocompatibility complex (MHC) alleles of the host, and typing reagents for the range of macaque class I alleles are not present, class I restriction was assessed by indirect methods. Brefeldin A (BFA; Sigma), which selectively blocks the presentation of peptides in association with MHC class I molecules and does not interfere with class II presentation of antigens, was used as previously described (22) to examine if the observed cytolytic responses were restricted to MHC class I. Briefly, target BLCL were pretreated with BFA (0.2  $\mu$ g/ml) for 30 min and maintained in BFA during infection with the rVV, incubation with  $^{51}$ Cr, and the lytic assay. The reversibility of blockade, an indication that BFA was not globally toxic to cells, was assessed by washing an aliquot of BFA-treated targets twice and resuspending the cells in fresh medium without BFA immediately prior to the lytic assay. Previous studies in our laboratory have affirmed that this dose of BFA has no effect on target recognition by class II-restricted CD4<sup>+</sup> T cells (22).

**T-cell proliferative responses.** PBMC were incubated for 6 days in quadruplicate replicate cultures at  $10^5$  cells per well in a total of 200  $\mu$ l in 96-well U-bottom plates containing either SIV<sub>MNE</sub> gp160 purified from a recombinant baculovirus (10  $\mu$ g/ml) or  $10^4$  autologous,  $\gamma$ -irradiated BLCL infected with an rFPV expressing SIV<sub>MAC</sub> *gag/pol*. Unstimulated control responses were determined by incubating PBMC with either baculovirus protein or autologous BLCL infected with rFPV<sub>lacZ</sub>. Wells were pulsed with 2.5  $\mu$ Ci of [ $^3$ H]thymidine 18 h before harvesting. Proliferation is expressed as stimulation index, measured as mean [ $^3$ H]thymidine incorporation of cells stimulated with antigen/mean incorporation in unstimulated control cells.

## RESULTS

**Protection of SIV-vaccinated macaques.** Macaques were immunized with an rVV expressing either SIV *env* or SIV *gag/pol*, boosted with the respective recombinant SIV protein, and challenged with SIV as described in Table 1. Vaccinated animals were considered protected and selected for study if they did not seroconvert to SIV (i.e., there was no increase in titers of SIV antibodies), exhibited no anamnestic antibody response postchallenge to the SIV vaccine protein, had no recoverable infectious virus (i.e., no coculture taken at any time point was positive for SIV), and remained healthy with normal numbers of CD4<sup>+</sup> T cells during the monitoring period postchallenge of 60 to 160 weeks. In contrast to these vaccinated animals, 11 of 11 unvaccinated control animals concurrently challenged with SIV<sub>MNE</sub> reproducibly yielded SIV by coculture of PBMC at multiple time points, seroconverted to several SIV proteins, and exhibited depletion of CD4<sup>+</sup> cells with progression to simian AIDS as previously reported following challenge with this virus (14, 23).

Three vaccinated macaques that were protected from challenge were available for analysis (Table 1). PBMC were obtained at multiple time points and tested for SIV DNA by PCR (14). Two animals were negative at all time points tested. PBMC from the third animal (animal 91278) had low but detectable SIV DNA by PCR in three separate assays of a specimen obtained at 2 weeks postchallenge (Table 1). With radiolabeled primers and known amounts of control SIV DNA, this reflected approximately 40 copies of SIV DNA per  $\mu$ g of input DNA, compared with a mean of 2,000 copies of SIV DNA per  $\mu$ g of input DNA from three unvaccinated animals identically challenged and evaluated at 2 weeks postinfection. The assay used had a sensitivity of approximately one to three copies of SIV per  $\mu$ g of input DNA. However, PBMC were consistently negative at all other time points tested, and lymph node mononuclear cells from a biopsy obtained at 20 weeks postchallenge were also negative. All unvaccinated challenged control animals studied had SIV DNA detected by PCR in peripheral blood and lymph node mononuclear cells at every time point examined (13, 14). Thus, molecular analysis sug-

gested that despite the absence of recoverable virus by culture or a seroconversion to SIV antigens, transient low-level infection may have occurred in at least one of the three macaques that exhibited vaccine-induced protection.

**CTL responses postchallenge.** CTL responses to SIV were assessed by stimulating PBMC in vitro for two 1-week cycles with autologous stimulator cells infected with rFPV expressing SIV proteins. These recombinant viruses were used to facilitate detection of CTL responses to SIV proteins, since they can be used as shuttle vectors to obtain expression of individual SIV genes in stimulator cells without inducing confounding CTL responses to vaccinia proteins present in animals previously immunized with an rVV, as previously described (22). In seven of seven unvaccinated SIV<sub>MNE</sub>-infected macaques evaluated between 4 and 104 weeks postchallenge, CTL responses to both SIV *env*- and *gag/pol*-encoded proteins were detected (Table 2). By contrast, no SIV-specific CTL responses were detected following in vitro stimulation (as defined by specific lysis of less than 10% with targets expressing SIV<sub>MNE</sub> *env* or *gag/pol* at E/T ratios of 6.25:1 to 50:1) in five of five unvaccinated healthy macaques not challenged with SIV (22), and no SIV-specific CTL were detected prior to SIV challenge using this method of in vitro stimulation in eight of eight animals vaccinated by the rVV-based SIV subunit regimen.

CTL responses from the three vaccinated and protected macaques were evaluated between 10 and 80 weeks postchallenge (Table 2). Animals 87217 and 90071, despite having been vaccinated with preparations that contained only SIV *env*, had demonstrable CTL responses to SIV *gag/pol* at multiple time points from 10 to 54 weeks postchallenge. Animal 91278, despite having been immunized only to SIV *gag/pol*, had CTL reactive to SIV *env* at 12 and 24 weeks post-SIV challenge, the two time points tested, as well as a weak response to SIV *gag/pol*. Thus, all three vaccine-protected animals developed, following SIV challenge, CTL reactive to SIV proteins expressed by cells infected with the virus but not by the vaccine.

**pCTL frequencies postchallenge.** pCTL frequency assays were used to estimate the relative strengths of the observed CTL responses. By using methods that we previously described (21), fresh PBMC were obtained at several time points relative to virus challenge and stimulated with autologous SIV *env*- or *gag/pol*-expressing cells plus rIL-2 in 24 replicates of increasing cell dilutions. Cells from each well demonstrating growing cells were assayed against a panel of autologous target cells. Animal 90071, previously vaccinated only to envelope, demonstrated more than a 10-fold increase in the frequency of pCTL reactive to *gag/pol* recovered following challenge compared with the prechallenge levels of <1 pCTL per  $10^6$  PBMC, which is similar to levels in naive animals. Animal 91278, previously vaccinated only to *gag/pol*, demonstrated increased frequency of pCTL postchallenge to both *env* and *gag/pol* (Table 3 and Fig. 1). The frequencies of pCTL postchallenge to the SIV proteins that had not been present in the vaccine regimen were similar to the frequencies detected in an unvaccinated, SIV-infected macaque (animal 91324) during the first year following SIV<sub>MNE</sub> challenge.

**Phenotype of CTL.** The contributions of CD4<sup>+</sup> and CD8<sup>+</sup> T cells to the lytic activity detected postchallenge were assessed by fractionating the T-cell population responding to stimulation with cells expressing SIV *gag/pol* into subsets by positive and negative selection on plates coated with  $\alpha$ CD8 MAb and then testing the subpopulations in a chromium release assay. Enrichment for CD8<sup>+</sup> T cells of the SIV *gag/pol*-reactive T cells elicited postchallenge from animal 90071 (the prior recipient of an envelope-based vaccine) resulted in an increase in cytolytic activity compared with the unfractionated population

TABLE 2. SIV-specific CTL responses before and after SIV challenge<sup>a</sup>

Animal no.	Vaccine immunogen	Target cell infection	% Specific lysis of target cells at indicated time post-SIV challenge <sup>b</sup>			
			30 wk	34 wk	54 wk	80 wk
87217	SIV <i>env</i>	Vac	7	4	4	3
		Vac- <i>env</i>	8	7	9	6
		Vac-gag/pol	17	21	15	6
90071	SIV <i>env</i>	Vac	-10 wk	-2 wk	10 wk	32 wk
		Vac- <i>env</i>	2	3	2	1
		Vac-gag/pol	5	7	6	5
91278	SIV gag/pol	Vac	1	0	22	16
		Vac- <i>env</i>	-2 wk	12 wk	24 wk	
		Vac-gag/pol	4	4	3	
90099 <sup>c</sup>	SIV <i>env</i>	Vac	2	0		
		Vac- <i>env</i>	1	2		
		Vac-gag/pol	2	0		
91324 <sup>d</sup>	None	Vac	26 wk	32 wk	62 wk	80 wk
		Vac- <i>env</i>	0	3	4	1
		Vac-gag/pol	12	9	15	11
			24	18	25	32

<sup>a</sup> Procedures for generating CTL and testing lytic activity are described in Materials and Methods. The data presented represent the results of experiments performed at an E/T ratio of 50:1.

<sup>b</sup> Boldface numbers represent demonstrable specific lytic activity as reflected by lysis of >10%. Negative sign preceding weeks represents weeks prior to challenge.

<sup>c</sup> Animal 90099 is representative of eight vaccinated control macaques that were not challenged with SIV.

<sup>d</sup> Animal 91324 is representative of seven evaluated control macaques not vaccinated but challenged with SIV.

of cells (Table 4). By contrast, cytolytic activity was not detected in the subpopulation enriched for CD4<sup>+</sup> T cells.

Limiting-dilution cultures were used to assess pCTL frequency to SIV proteins in animal 91278, the recipient of a gag/pol-based vaccine, and both SIV *env*- and gag/pol-specific pCTL were detected at 12 weeks postchallenge. The experiment was repeated at 24 weeks postchallenge, but in this instance subpopulations of PBMC enriched for either CD4<sup>+</sup> or CD8<sup>+</sup> cells were plated in limiting dilution. Cytolytic precursors suggestive of a primed response, as observed at week 12, were detected in plated cells derived from the CD8<sup>+</sup> fraction of PBMC, whereas the CD4<sup>+</sup>-enriched fraction yielded CTL with a frequency lower than that observed in the unfractionated populations prior to challenge (Table 3). Thus, the detected lytic activity appeared to reside in the CD8<sup>+</sup> population.

Target recognition by CD8<sup>+</sup> CTL requires the endogenous expression of viral gene products in infected cells for processing and presentation in association with class I molecules. Since panels of macaque cells typed for class I are not available, the requirement for recognition of peptides in association with class I molecules on target cells by the demonstrated CTL was assessed by treatment of targets with BFA. This compound selectively and reversibly interferes with presentation of peptides from newly synthesized proteins with class I molecules by blocking egress of class I molecules from the Golgi complex (22, 29, 41). BFA treatment of target cells blocked target recognition by SIV gag/pol-specific CTL derived from animal 90071 (Table 4). Moreover, removal of the BFA by washing the targets prior to the cytolytic assay resulted in restoration of the sensitivity of these targets to lysis, consistent with a re-

TABLE 3. Frequency of pCTL to SIV proteins before and after SIV challenge<sup>a</sup>

Animal no.	Vaccine immunogen	Target cell infection	pCTL/10 <sup>6</sup> PBMC at indicated time post-SIV challenge (95% CI) <sup>b</sup>			
			-2 wk	10 wk	34 wk	52 wk
90071	<i>env</i>	Vac	0.3 (0.1-0.9)	0.7 (0.1-1.3)	1.1 (0.4-1.9)	0.6 (0.1-1.0)
		Vac- <i>env</i>	0.7 (0.3-1.2)	1.3 (0.6-2.0)	1.3 (0.6-2.0)	1.0 (0.4-1.6)
		Vac-gag/pol	0.4 (0.1-0.8)	5.6 (3.8-7.5)	5.2 (3.2-7.3)	3.1 (1.9-4.4)
91278	gag/pol	Vac	-2 wk	12 wk	24 wk (CD4 <sup>+</sup> cells) <sup>c</sup>	24 wk (CD8 <sup>+</sup> cells)
		Vac- <i>env</i>	0.8 (0.1-1.5)	0.8 (0.4-1.2)	0.7 (0.2-1.2)	1.2 (0.5-1.8)
		Vac-gag/pol	0.8 (0.4-1.1)	3.8 (2.5-5.1)	0.6 (0.1-1.1)	3.8 (2.4-5.2)
91324	None	Vac	1.0 (0.5-2.0)	4.1 (2.8-5.5)	0.5 (0.1-1.0)	6.9 (4.7-9.2)
		Vac- <i>env</i>	32 wk	66 wk	84 wk	
		Vac-gag/pol	0.6 (0.2-0.9)	1.1 (0.5-1.8)	0.8 (0.1-1.4)	
			4.0 (2.7-5.3)	3.3 (2.0-4.6)	3.0 (1.8-4.3)	
			6.0 (4.0-8.1)	6.3 (5.2-7.5)	5.5 (4.1-6.9)	

<sup>a</sup> Limiting-dilution assays were performed as described in Materials and Methods. pCTL frequencies and 95% confidence intervals (CI) assume a single-hit model with a Poisson distribution and were calculated by  $\chi^2$  minimization (40).

<sup>b</sup> Boldface numbers represent pCTL frequencies to SIV proteins more than 3 standard deviations greater than concurrently measured vaccinia virus-specific pCTL frequencies.

<sup>c</sup> For animal 91278, fresh PBMC obtained at 24 weeks postinfection were separated into CD4- or CD8-enriched cells, and the cell fractions were assayed separately.

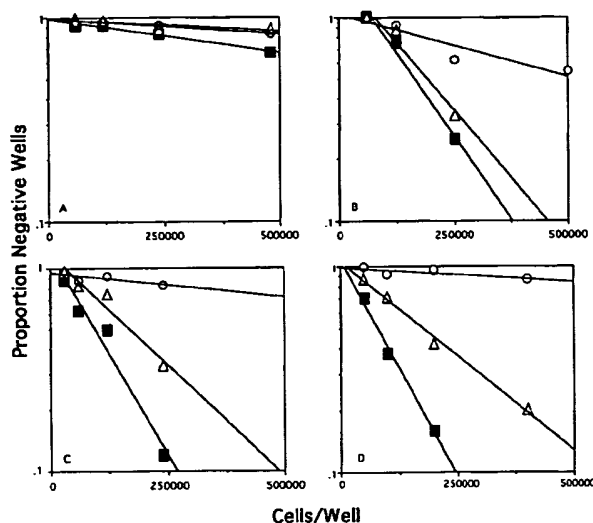


FIG. 1. Analysis of pCTL frequency by limiting-dilution culture of PBMC for cells reactive with SIV proteins. PBMC were obtained from a macaque vaccinated to SIV *gag/pol* (animal 91278) prechallenge (A), at 12 weeks postchallenge with SIV<sub>MNE</sub> (B), and at 24 weeks postchallenge, at which time CD8<sup>+</sup> cells were selected from the PBMC prior to plating at limiting dilution (C). PBMC obtained 62 weeks postchallenge from an unvaccinated, SIV<sub>MNE</sub>-infected animal (animal 91324) were assayed for comparison (D). Lytic activity was assessed against a panel of autologous BLCL targets infected with vaccinia virus vectors and expressing either no SIV proteins (open circles) or proteins encoded by SIV *env* (open triangles) or SIV *gag/pol* (closed squares).

quirement for the presence of BFA to prevent egress of class I molecules rather than a nonspecific toxic effect on the cells. Previous experiments have demonstrated that this dose of BFA does not interfere with recognition of target cells by class II-restricted CD4<sup>+</sup> T cells. Thus, the cytolytic activity detected in these animals appeared to be largely mediated by CD8<sup>+</sup> T cells recognizing SIV antigens in association with class I molecules.

**Clonal analysis of CTL response postchallenge.** CTL clones were generated following limiting-dilution culture to permit definitive characterization of the cells mediating lytic activity. Four SIV *gag/pol*-specific CTL clones were isolated from PBMC obtained from animal 90071 (an SIV *env*-vaccinated animal that resisted SIV challenge) 10 weeks following SIV challenge. These clones were CD3<sup>+</sup> CD4<sup>+</sup> CD8<sup>+</sup> CD16<sup>+</sup> by FACS analysis and demonstrated significant SIV *gag/pol*-specific lytic activity at a low E/T ratio (10:1) (Table 5). Analysis of lysis of targets generated from an unrelated SIV-infected animal demonstrated that the lytic activity was restricted to autologous and not allogeneic (presumably mismatched) targets.

**Analysis of proliferative T-cell responses to SIV proteins in vaccinated macaques prior to challenge with SIV.** The efficient generation of CD8<sup>+</sup> responses post-challenge with SIV would be facilitated by the presence of a CD4<sup>+</sup> helper response. We have previously reported that rVV-based lentivirus vaccine regimens, such as those used in this study, induce CD4<sup>+</sup> T-cell proliferative responses (5, 18). PBMC specimens obtained prechallenge were available for study from two of the three vaccinated animals protected from SIV challenge, and the presence of vaccine-induced CD4<sup>+</sup> T cell proliferative responses prior to challenge was analyzed. PBMC were stimulated with either purified soluble envelope protein or autologous cells expressing *gag/pol*-encoded proteins. Cells from animal 90071,

which had been immunized to SIV envelope, proliferated in response to stimulation with SIV envelope but not SIV *gag/pol* (Table 6). Fractionation of the PBMC to enrich for CD4<sup>+</sup> and CD8<sup>+</sup> populations demonstrated that the responding population was derived from the CD4<sup>+</sup> subset. Cells from animal 91278, which had been immunized to SIV *gag/pol*, in contrast, proliferated in response to SIV *gag/pol*-encoded proteins but not SIV envelope, and the responding population was again derived from the CD4<sup>+</sup> subset. Although PBMC obtained prior to challenge were not available from animal 87217, we have previously reported that SIV envelope-specific T-cell proliferation was detectable in this animal prior to challenge (14, 18). These results demonstrate that although CD8<sup>+</sup> T-cell responses to the vaccine immunogen were no longer detectable at the time of challenge, CD4<sup>+</sup> proliferative responses remained detectable.

## DISCUSSION

Our data indicate that in macaques previously immunized with an SIV subunit vaccine, SIV-specific, class I-restricted, CD8<sup>+</sup> CTL were induced following challenge with infectious SIV to SIV proteins expressed in infected cells but not the subunit vaccine, despite protection from the establishment of SIV infection. These CTL responses postchallenge were observed in all three of the vaccinated macaques studied that were protected from challenge. The presence of such CTL responses to SIV proteins expressed in infected cells is not likely to reflect chance cross-reactivity with environmental antigens, since CTL responses to these proteins were neither detectable prior to challenge in the 2 of 3 macaques in which prechallenge PBMC were available for analysis nor detectable in PBMC from 13 control macaques studied prechallenge. Since cell-free virus rather than cell-associated virus was used for the SIV challenges in this study, the observed induction of CTL specific for proteins not incorporated in the subunit vaccine could not reflect recognition of cells expressing SIV genes in the challenge inoculum (11). Therefore, the detection of these CTL suggests that SIV infection of host cells with expression of the relevant SIV proteins occurred postchallenge.

TABLE 4. Analysis of phenotype and requirement for target presentation of antigenic peptide in association with class I molecules<sup>a</sup>

Target cell treatment <sup>b</sup>	Target cell infection	% Specific lysis by effector cell population		
		Unseparated	CD4 enriched	CD8 enriched
No BFA	Vac	3	3	2
	Vac- <i>gag/pol</i>	18	4	23
BFA	Vac	2	2	0
	Vac- <i>gag/pol</i>	1	3	1
BFA with washout	Vac	2	3	2
	Vac- <i>gag/pol</i>	16	2	18

<sup>a</sup> PBMC were obtained 16 weeks following SIV challenge of animal 90071, which had previously been immunized to SIV *env* and was protected from the challenge. The PBMC were stimulated in vitro with autologous cells infected with rFPV vectors, and the resultant effector cells were tested for cytolytic activity either as unseparated cells or following enrichment for CD8<sup>+</sup> or CD4<sup>+</sup> T cells.

<sup>b</sup> Target cells were either not treated to interfere with class I presentation (no BFA), exposed to BFA throughout target cell preparation, including labeling with <sup>51</sup>Cr, infection with the rVV, and the cytolytic assay, or treated with BFA during target cell preparation but with the BFA washed out immediately prior to assay (BFA with washout). The cytotoxicity data were generated with an E/T ratio of 50:1.

TABLE 5. Specificity of CD8<sup>+</sup> CTL clones isolated from SIV-challenged macaques<sup>a</sup>

Clone (animal no.)	Target cell infection	% Specific lysis of target cells	
		BLCL from animal 90071	BLCL from animal 91324
9D3 (90071)	Vac	0	1
	Vac-gag/pol	34	0
1D11 (91324)	Vac	2	2
	Vac-gag/pol	1	41

<sup>a</sup> CD8<sup>+</sup> T-cell clones specific for SIV gag/pol were isolated by limiting dilution from reactive T-cell lines generated from animal 90071 (an SIV env-vaccinated animal) with PBMC obtained 10 weeks postchallenge and from animal 91324 (an unvaccinated animal) with PBMC obtained 92 weeks postinfection. The CTL clones were tested for lysis of autologous and allogeneic target cells infected with Vac or Vac-gag/pol. One representative CD8<sup>+</sup> clone from each of the animals is presented. The data were generated at an E/T ratio of 10:1.

As only limited numbers of macaques were available for evaluation, it is not possible to determine from this study if subunit vaccination can induce sterilizing immunity, but the results suggest that such immunity is not obligatory for protection.

The apparent SIV infection in the vaccine-protected animals was likely transient and limited to below the levels of detection (which was one to three copies of SIV per µg of input DNA) rather than persistent, as suggested by several findings in these animals distinct from those observed in challenged animals in which infection becomes established: the lack of recoverable infectious virus, the absence of a detectable serologic response to SIV postchallenge, and the long-term health of the animals. Low copy numbers of SIV proviral DNA (40 copies per µg of DNA) were detected in PBMC from one of the three animals at 2 weeks postchallenge, but this positive signal was no longer evident (i.e., <1 to 3 copies per µg of DNA) at subsequent time points, including PCR of a lymph node biopsy at 20 weeks. These PCR results provide further confirmation for the CTL data showing that host cells can be transiently infected following challenge. Since no samples were evaluated in any of the animals prior to 2 weeks postinfection, it is formally possible that PCR analysis of either PBMC or lymph nodes might provide evidence for a transient SIV infection in additional macaques if samples were examined at earlier and more frequent time points.

The CTL to nonvaccine SIV antigens detected in this study were present to approximately 1 year postchallenge; indeed, the challenge with live SIV, despite protection, produced a readily detectable and relatively durable CTL response, whereas immunization with the vaccinia virus-SIV recombinants had produced weak or nondetectable responses. Although earlier studies had suggested that the persistence of CTL requires the presence of at least a low level of antigen (10,

30), more recent studies with transfer of murine CD8<sup>+</sup> CTL have firmly established that viral antigen is not required for the persistence of virus-specific CD8<sup>+</sup> CTL responses (12, 25, 28). This finding is consistent with a recent study of humans immunized with vaccinia virus, in which CD8<sup>+</sup> CTL were shown to persist for decades in the absence of apparent viral persistence and replication (7). In studies of HIV-specific CTL in uninfected infants born to HIV-infected mothers (in whom infection of the infants was assessed by DNA PCR), HIV-specific CTL activity was detected up to at least 5 months of age and in one case up to 35 months of age (4, 35).

It is surprising that despite this apparently transient SIV infection that elicited SIV-specific CD8<sup>+</sup> CTL responses, no primary or anamnestic antibody responses to SIV proteins developed postchallenge. Since these animals had no neutralizing antibody activity at the time of challenge, and indeed the protective gag-subunit vaccine has no potential to elicit such antibodies, the results suggest that host cellular immune responses likely played a critical role in rapidly controlling the magnitude of in vivo viral replication and mediating efficient viral clearance.

Although the CD8<sup>+</sup> CTL responses elicited post-SIV challenge provide a marker for the occurrence of transient infection postchallenge, the precise mechanism(s) by which immune responses induced by subunit vaccines can promote rapid and complete eradication of cells infected following exposure to SIV have not yet been defined (37). The vaccine regimens examined in this study, as well as results with similar vaccine regimens using rVV priming and boosting with recombinant protein, have been shown to induce high CD4<sup>+</sup> SIV-reactive helper T cells and CD8<sup>+</sup> CTL, but in general the CD8<sup>+</sup> responses detected postimmunization in the peripheral blood compartment have been only weak and transient (5, 18, 20). It is possible that the primed CD4<sup>+</sup> T cells observed to persist postvaccination rapidly generate following SIV challenge an inflammatory response, boost existing SIV-reactive primed CD8<sup>+</sup> T cells that had been induced by vaccination, and facilitate efficient priming of naive CD8<sup>+</sup> T cells to the multiple antigens expressed in SIV-infected cells. Moreover, responding CD4<sup>+</sup> T cells can release cytokines such as tumor necrosis factor alpha that both promote direct lysis of virally infected cells and activate viral gene transcription, thereby preventing latency and rendering infected cells susceptible to elimination by the evolving CD8<sup>+</sup> CTL responses (1, 2). Future studies will need to be designed to decipher the obligate contributions of the distinct components of the immune response to protection so that vaccine regimens can be developed to preferentially induce and maintain such responses. However, the present data provide encouragement for vaccine development by demonstrating that an effective vaccine to lentiviruses may not be required to induce persistent sterilizing immunity but rather

TABLE 6. T-cell proliferative responses to SIV proteins in SIV-vaccinated macaques prior to challenge with SIV

Animal no.	Vaccine immunogen	SI in indicated responding population <sup>a</sup>					
		PBMC		CD4-enriched cells		CD8-enriched cells	
		env	gag/pol	env	gag/pol	env	gag/pol
90071	SIV env	12.2	0.9	16.8	1.2	1.5	0.9
91278	SIV gag/pol	1.1	3.3	1.2	5.9	1.0	1.2

<sup>a</sup> Responding populations were either unfractionated PBMC or PBMC enriched for CD4<sup>+</sup> or CD8<sup>+</sup> cells by positive and negative selection on flasks coated with αCD8 MAb as described in Materials and Methods. The PBMC were obtained from both animals 2 weeks prior to challenge with SIV. Cells were stimulated with either purified SIV envelope protein produced in recombinant baculovirus or autologous BLCL stimulator cells infected with rFPV expressing SIV gag/pol. The stimulation indices (SI) in response to either control baculovirus proteins or autologous BLCL infected with rFPV<sub>lacZ</sub> compared with no stimulation were <1.5 in both animals.

may need only enhance the efficiency and speed with which the host can recognize and eliminate infected cells.

#### ACKNOWLEDGMENTS

We thank V. Stallard for technical help, D. Panicali for provision of rFPV constructs, and J. Factor for manuscript preparation.

This work was supported by NIH grants AI27757, AI26503, RR00166, and AI28065. S.J.K. is a recipient of a Commonwealth AIDS Research Grant (Australia) and a Harkness fellowship (Commonwealth Fund, New York).

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